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Specific adaptation by *Mycosphaerella graminicola* to a resistant wheat cultivar

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Three cultivars of winter bread wheat (Gene, Madsen and Stephens) were each inoculated as seedlings in the greenhouse with seven or eight individual isolates of *Mycosphaerella graminicola* collected in 1997 from each of the same cultivars in the field. Isolates collected from Gene were virulent to all three cultivars, while isolates obtained from Madsen and Stephens were virulent to those two cultivars and, in all but one case, avirulent to Gene. At its release in 1992, Gene was resistant to *M. graminicola*, as indicated by both field observations and greenhouse tests, but by 1995 its resistance had substantially deteriorated. This indicated that its resistance was vertical (*sensu* Vanderplank) or race-specific, and that commercial cultivation of Gene rapidly selected for strains in the local *M. graminicola* population that were specifically adapted to overcome its resistance.

Keywords: *Mycosphaerella graminicola*, physiologic specialisation, septoria tririci blotch, *Triticulm aestivum*, winter bread wheat

Introduction

Septoria tritici blotch is a foliar disease of durum and bread wheat caused by *Mycosphaerella graminicola* (anamorph *Septoria tritici*), which inflicts major losses on wheat crops in areas of relatively high rainfall and moderate temperatures. Severity of septoria tritici blotch appears to be increasing in many parts of the world (Scharen, 1999), including the Willamette Valley of Oregon, USA (Mundt *et al.*, 1999).

Host plant resistance is the method of choice for control of septoria tritici blotch. Whether *M. graminicola* has specific interactions with its hosts is important in breeding for resistance, and has been extensively debated. There is widespread agreement that it is specialized at the level of the host species, on bread or durum wheat (Eyal *et al.*, 1973; Eyal *et al.*, 1985; Saadaoui, 1987; van Ginkel & Scharen, 1988; Kema *et al.*, 1996a). Three early reviews (Shipton *et al.*, 1971; King *et al.*, 1983; Nelson & Marshall, 1990) suggested that *M. graminicola* probably does not possess cultivar specificity. In these three reviews, the total evidence cited against physiological races and specificity consists of two abstracts, one reference to unpublished data and two published papers. However, numerous recent studies have yielded evidence of differential cultivar–isolate interactions including several that used only same-species isolates and testers (i.e. bread-wheat isolates on bread wheats) (Ahmed *et al.*, 1995; Ahmed *et al.*, 1996; Kema *et al.*, 1996b; Kema & van Silfhout, 1997). Kema *et al.* (1996b) and Kema & van Silfhout (1997) inferred gene-for-gene interaction in the *M. graminicola*–wheat pathosystem because of significant interaction mean-square values in analysis of variance and covariance, and cluster analyses indicating substantial genetic variance for virulence and resistance among isolates and cultivars.

If isolate × cultivar and gene-for-gene interactions do occur, what is the practical significance for the durability of resistance? Previous studies of *M. graminicola*–wheat interaction have involved isolates and cultivars that were unrelated to each other; often the choice of isolates was apparently arbitrary. Significant cultivar–isolate interactions in these experiments tell only of the potential for specific adaptation, not whether such adaptation has in fact occurred or will occur. Experiments in which isolates have been selected on the cultivars studied can reveal actual patterns of adaptation to host.

This paper describes an experiment with *M. graminicola* isolates collected in 1997 from three cultivars with different levels of resistance, and tested as single isolates on the same cultivars in the greenhouse. This work provides evidence that the 1992 introduction of a cultivar with resistance to septoria tritici blotch was soon followed by the evolution and retention in the local *M. graminicola* population of strains with specific virulence to that cultivar, indicating that its resistance was vertical (*sensu* Vanderplank, 1968) or race-specific.

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Materials and methods

The experiment was conducted in 1998 with wheat seedlings grown in the greenhouse and monopycnidial isolates of *M. graminicola* collected in July 1997. Pots of wheat seedlings (testers) were inoculated with individual isolates, and the experimental unit was the pot.

The experiment was a factorial of tester cultivar and isolate. The soft white winter wheat cultivars Gene (P.I. 560129), Madsen (P.I. 511673) and Stephens (C.I. 017596) served both as sources of isolates in the field and also as tester cultivars in the greenhouse. At its release in 1992, Gene was highly resistant to *M. graminicola*, but that resistance has declined markedly since then (Mundt *et al.*, 1999). Madsen has moderate resistance to septoria tritici blotch, and Stephens is highly susceptible.

There were 22 monopycnidial isolates *of M. graminicola* (seven each derived from Gene and Stephens, and eight from Madsen). The isolates had been collected at random from 3×22 m experimental plots at the Hyslop Crop Science Field Research Laboratory in Corvallis, Oregon. The Gene isolates came from five different Gene flag or F_1 leaves (in some cases flag leaves were difficult to isolate from because of low levels of infection), and the Madsen and Stephens isolates originated from four different flag leaves of those cultivars, respectively. Thus the probability that two isolates from a particular cultivar were genetically identical was extremely low, as even pycnidia from different lesions on the same leaf are highly likely to be genetically distinct (McDonald & Martinez, 1990). The experiment was replicated three times.

In each replication, 10 cm pots were planted with ≈15 seeds of each cultivar. The pots were fertilized with Osmocote 18-6-12 extended time-release fertilizer (Scott-Sierra Horticultural Products Co., Marysville, USA) at a rate of about 1 g per pot at seeding, and watered as needed. Soon after seedling emergence the pots were thinned to a density of 10 plants per pot.

M. graminicola isolates were maintained on refrigerated silica gel and yeast-malt agar, and inoculum was prepared by growing the isolates for 5–7 days on yeast-malt agar slants. The conidia were suspended in distilled water and the suspension was adjusted to a concentration of approximately 1×10^6 spores per mL using a haemocytometer. For each isolate, 50 mL of the suspension was used to inoculate seedlings (one pot of each of the three cultivars) to the point of run-off. Pots were inoculated at 21 days after planting, and placed in a mist chamber with high relative humidity for 96 h. They were then returned to the greenhouse bench, arranged in random order within each block and with even spacing on the bench, and watered by drip irrigation to promote constant canopy humidity. At 21 days after inoculation, symptoms of *S. tritici* leaf blotch were assessed. The third leaf from the bottom of each plant was assayed for percentage of leaf area covered by *M. graminicola* lesions, irrespective of pycnidial density. A mean percentage diseased leaf area (%DLA) was calculated for the approximately 10 plants in each pot.

Some researchers have assayed necrotic leaf area (Eyal *et al.*, 1985; van Ginkel & Scharen, 1987); others have measured pycnidial coverage (Rosielle, 1972; Eyal *et al.*, 1973; Kema *et al.*, 1996a; Kema & van Silfhout, 1997) or percentage of leaf area with necrotic lesions containing pycnidia (Somasco *et al.*, 1996). In the present experiments percentage leaf area occupied by *S. tritici* blotch lesions was recorded, rather than either necrotic leaf area or pycnidial density, as a measure of disease. Some of the lesions observed contained few or no pycnidia. It was possible to distinguish a lesion, even if it did not contain pycnidia, from other necrotic leaf tissue, particularly if the necrosis on water controls was used as a reference point and if trials were conducted during the period from November to March. This choice was based on observations that diseased leaf area proportions accurately reflected the susceptibility of cultivars in the field, and that naked-eye assessments of lesion area seemed more accurate and reproducible than those of pycnidial coverage.

As a result of the size of this experiment, the assessment of symptoms in each replication had to be conducted over 2 consecutive days. To account for disease increase from the first to the second assessment day, between four and eight treatments in each replication were assessed on both days, and the percentage change in diseased leaf area was calculated. A linear regression model was used to adjust day 1 means to be equivalent to day 2 means for each pot.

A previous greenhouse experiment using similar methods was conducted in 1994 with the same tester cultivars and isolates that had been collected in the field in 1992. The only differences in method were that the experiment had four replicates; 25 mL instead of 50 mL of spore suspension were applied to each group of four pots (the three reported here plus a fourth); and the second rather than the third leaf from the bottom was assessed.

Statistical analyses

Data were subjected to analysis of variance. As disease severity on the water controls was negligible (Table 1), these values were excluded from the analysis in order to avoid underestimating the mean square for error. To improve homogeneity of variance, the data were $\log_e$-transformed (with the quantity 1·0 added to all original values). Fixed effects were origin, tester cultivar, and their interaction. Replicate, isolate within origin, and interactions involving those two effects were treated as random. The origin–tester interaction was dissected with linear contrasts to determine if isolates collected from Gene were specifically adapted to that cultivar. As an additional test of specific adaptation, Pearson's chi-square test with Yates's continuity correction was

Table 1 Percentage diseased leaf area of greenhouse-grown wheat seedlings inoculated with *Mycosphaerella graminicola* isolates collected in 1997[a]

Cultivar of origin & isolate number	Tester cultivar			
	Gene	Madsen	Stephens	Mean[b]
Gene				
1	19·9	26·4	9·4	18·6
2	3·4	18·5	16·7	12·8
3	13·0	29·5	23·4	21·9
4	11·2	17·9	29·6	19·5
5	43·6	63·4	63·4	56·8
6	21·1	32·9	33·0	29·0
7	37·2	61·3	56·6	51·7
Madsen				
8	0·9	77·1	56·0	44·7
9	3·0	7·4	11·5	7·3
10	4·2	30·6	28·4	21·0
11	10·7	38·0	31·5	26·8
12	1·0	30·1	28·7	19·9
13	1·3	30·1	22·6	18·0
14	0·2	19·6	7·8	9·2
15	0·7	15·1	13·4	9·8
Stephens				
16	3·3	22·7	24·7	16·9
17	1·0	13·2	7·9	7·4
18	0·4	31·0	21·7	17·7
19	2·5	31·4	24·9	19·6
20	2·7	22·0	16·1	13·6
21	0·7	15·5	14·3	10·2
22	2·2	36·1	28·3	22·2
Grand mean	8·4	30·5	25·9	21·6
Water controls	0·3	1·1	1·6	1·0

[a]Experiment conducted in 1998.[b]Means are the untransformed percentage diseased leaf area over four replications. Statistical analyses were conducted on $\log_e$-transformed data.

performed on a contingency table in which isolates were classified as virulent or avirulent on Gene, and originating either from Gene or from Madsen/Stephens. Origin and tester means were separated using Fisher's protected least significant difference test.

To determine the importance of the isolate–tester interaction, the three 1997 replicates were analysed separately because of a significant interaction between isolates and replicates. For this assessment, the intraclass correlation (Kuehl, 1994), which measures the similarity of observations within groups compared to observations between groups, was calculated using untransformed data. In this case the intraclass correlation coefficients indicate the correlation in values among testers inoculated with the same isolate. The intraclass correlation coefficient is the ratio

$$[\sigma^2_{\text{isolate(origin)}}]/[\sigma^2_{\text{isolate(origin)}} + \sigma^2_{\text{isolate(origin)} \times \text{tester}}].$$

Here the ratio can take on values from $-1/2$ $[-1/(\text{number of testers} - 1)]$ to 1. A ratio close to 1 indicates a strong correlation in disease readings among testers affected by the same isolate; that is, variation between isolates is more important than that of isolate–tester interaction. Conversely, a ratio toward the low end of the range indicates a lack of correlation in disease levels among cultivars inoculated with the same isolate; that is, the preponderance of variation can be attributed to the interaction of isolates and testers.

Results

Isolates applied to tester Gene can be clearly separated into virulent (>10% DLA) and avirulent (≤5% DLA) categories (Table 1). Of the seven 1997 isolates from Gene, only one caused less than 5% DLA on Gene, and the mean was 21·3% (Fig. 1). The Gene isolates were also strongly virulent to Madsen and Stephens, causing an average of 35·7 and 33·1% DLA, respectively. In

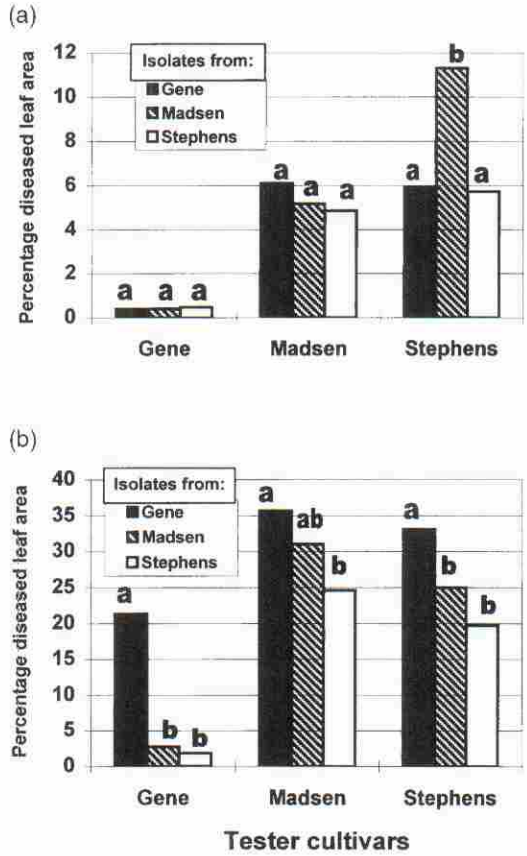

Figure 1 Mean percentage diseased leaf area caused by isolates of *Mycosphaerella graminicola* collected from each of three cultivars in the field and inoculated singly on each of the same cultivars as seedlings in the greenhouse. Within a tester, bars topped by the same letter are not significantly different at the 95% confidence level based on Fisher's protected least significant difference. (a) Two isolates were collected from each cultivar in 1992 and the experiment was performed in 1994. Values are means of four replications. (b) Seven or eight isolates were collected from each cultivar in 1997 and the experiment was performed in 1998. Values are means of three replications.

Table 2 Analysis of variance using $\log_e$-transformed percentage disease severity data from greenhouse inoculation of wheat seedlings with single isolates of *Mycosphaerella graminicola* derived from the same cultivars in the field in 1997

Source	d.f.	Mean square	*F*-value	*P*
Rep	2	11·465	5·466	0·1102
Origin[a]	2	13·438	4·965	0·0268
Isolate(origin)	19	3·069	2·755	0·0040
Tester	2	53·715	21·214	0·0051
Origin × tester	4	4·240	4·099	0·0316
Isolate(origin) × tester	38	0·518	1·420	0·0979

[a]Origin = cultivar from which isolates originated.

fact, some isolates from Gene were more virulent to Madsen and Stephens than were isolates from those cultivars (Fig. 1b). However, nearly all isolates from Madsen and Stephens were avirulent to Gene, causing mean percentage DLAs of 2·8 and 1·8%, respectively. Only one of those 15 isolates, number 11 derived from Madsen, was virulent to Gene (10·7% DLA).

The origin–tester interaction was significant ($P = 0·0316$) (Table 2). Linear contrasts designed to dissect the origin–tester interaction revealed no interaction between origins and the testers Madsen and Stephens, but very strong interaction between the origins and tester Gene versus testers Madsen and Stephens (Table 3). Chi-square analysis confirmed that there was a significant difference between the proportion of isolates from Gene that were virulent to Gene (six of seven) and the proportion of isolates from Madsen/Stephens that were virulent to Gene (one of 15) ($x^2 = 10·3445$, 1 d.f., $P = 0·0013$).

For the 1998 experiment, the point estimates of intraclass correlation were 0·621, 0·518 and 0·473 for replicates 1, 2 and 3, respectively. The 95% confidence intervals for those estimates were (0·376, 0·811), (0·252, 0·748) and (0·200, 0·718), respectively.

None of the isolates collected in 1992 was virulent to Gene (Table 4), and the origin–tester interaction was not significant (data not shown).

Discussion

These results indicate that, following commercial introduction of Gene, strains rapidly appeared in the local *M. graminicola* population that were adapted to overcome its resistance. These strains persisted in the population even when Gene no longer occupied a substantial share of the commercial wheat area (Fig. 2).

At its release in 1992, Gene was almost completely resistant to *M. graminicola* (Mundt *et al.*, 1995; Mundt *et al.*, 1999). Gene is an F_4-derived selection from a cross involving the resistant cultivar Cleo, which is believed to carry the septoria tritici blotch resistance gene *Stb4* (Kronstad *et al.*, 1994; Somasco *et al.*, 1996). In 1994 and 1995, Gene quickly gained popularity among commercial growers in Oregon (Fig. 2), and by 1995 it occupied over 15% of the Willamette Valley wheat area. The data in Fig. 2 are valley-wide means; the proportion of wheat area occupied by Gene was substantially higher in the south-central Willamette Valley, where the experiments were carried out.

By 1995 it was evident that *M. graminicola* was rapidly overcoming the resistance of Gene (Mundt *et al.*, 1999). Its susceptibility also to *Leptosphaeria nodorum* (anamorph = *Septoria nodorum*) was an additional liability, and planting of Gene apparently increased the prevalence of that pathogen in the Willamette Valley. Commercial production of Gene declined, and by 1997 the cultivar accounted for only 3·9% of Willamette Valley wheat cultivation.

Consistent with this history, Ahmed *et al.* (1995) detected avirulent reactions on Gene for 14 monopycnidial isolates, including six from Oregon, that were all collected prior to 1992. Further, bulk populations of 20 isolates collected from each of four cultivars, including Gene, in 1992 produced avirulent reactions on Gene (Ahmed *et al.*, 1996). Its field performance was consistently highly resistant prior to commercial production (Mundt *et al.*, 1995; Mundt *et al.*, 1999). The data in Table 4 indicate avirulence to Gene in the six isolates collected in 1992; while these data by themselves would be insufficient, they corroborate other evidence that *M. graminicola* was avirulent on Gene before 1992.

The 1997 isolates obtained from Gene were virulent to all three cultivars with one exception, while those from Madsen and Stephens were avirulent to Gene, again with one exception. Inspection of Fig. 1 and the linear contrasts (Table 3) illustrate that most of the origin–cultivar interaction resulted from differential interactions of isolates on Gene. Similarly, Kema *et al.* (1996b) found considerable variation among subsets of host cultivars and pathogen isolates as to whether they appeared to possess specific resistance/virulence factors.

The rapid decline of resistance in Gene suggests that it is under the control of a monogenic or oligogenic system. This report now provides a clear example of

Table 3 Linear contrasts to dissect the cultivar-of-origin × tester cultivar interaction for 1997 isolates of *Mycosphaerella graminicola* collected from three wheat cultivars in the field and inoculated singly on the same three cultivars as greenhouse-grown seedlings

Linear contrast	d.f.	Mean square	*F*-value	*P*
Madsen vs Stephens × Origin[a]	2	0·003825	0·16	0·8580
Madsen & Stephens vs Gene × Origin[a]	2	0·197871	8·03	0·0068

[a]Origin = cultivar from which isolates originated.

Table 4 Percentage diseased leaf area of greenhouse-grown wheat seedlings inoculated with *M. graminicola* isolates collected in 1992[a]

	Tester			
Cultivar of origin[b]	Gene	Madsen	Stephens	Mean[c]
Gene				
Isol. 1	0·33	8·15	5·78	4·75
Isol. 2	0·48	4·03	6·10	3·54
Madsen				
Isol. 3	0·43	4·60	6·35	3·79
Isol. 4	0·38	5·75	16·28	7·47
Stephens				
Isol. 5	0·43	3·45	3·98	2·62
Isol. 6	0·53	6·25	7·45	4·74
Grand mean	0·43	5·37	7·66	4·49
Water control	0·30	0·20	0·20	0·23

[a]Experiment was conducted in 1994.[b]Field reactions: Gene, highly resistant at the time isolates were collected; Madsen, moderately resistant; Stephens, highly susceptible.[c]Means are the untransformed percentage diseased leaf area over four replications. Statistical analyses were conducted on $\log_e$-transformed data.

rapid decline in resistance of a cultivar to *M. graminicola* that was reported as missing from the literature by Johnson (1992) and Kema *et al.* (1996b). It should be noted that in the Willamette Valley, *M. graminicola* has near-optimal conditions for rapid evolution in response to host characteristics. Epidemics vary from moderate to severe, the sexual stage is abundantly present during more than one part of its life cycle (Mundt *et al.*, 1999; C.C., unpublished results), and genetic variability of the pathogen is extremely high (Boeger *et al.*, 1993; Chen *et al.*, 1994). In contrast, environmental conditions are less favourable to septoria tritici blotch in the Central Valley of California; even so, the resistance of cultivars planted extensively there starting in the mid-1990s had already eroded significantly, although not completely, by 1998 (L. Jackson, University of California at Davis, personal communication). At least one of those cultivars probably bears the *Stb4* resistance gene.

While the sample of 22 isolates was limited in size, it was probably adequate because of the high level of consistency in the response of Gene to isolates from each cultivar. Isolates were either virulent or avirulent, and isolates from the two other cultivars performed almost uniformly on Gene.

Average disease levels were considerably higher in the 1998 experiment than in the small experiment performed in 1994. The difference could result from environmental conditions in the greenhouse, a general increase in aggressiveness of the pathogen over the intervening years, or both. Isolates from 1992 and 1997 were not compared in the same test, and no claims are made about the relative aggressiveness of the two sets of isolates. A simultaneous test of both sets of isolates, while interesting, would address a different question from the main focus of this research, and would also introduce the confounding factor of differential periods of maintenance of the pathogen in culture. The data from the 1994 experiment simply help to establish the baseline of avirulence of isolates to Gene, which is amply confirmed by field data and other greenhouse studies.

The relative disease levels on the 1998 greenhouse testers did not mirror those of the same cultivars in the field (disease was, on average, most severe on Madsen, while Stephens is the most susceptible of the three in the field). One possible explanation is that the set of isolates was not perfectly reflective of the population as a whole. Another explanation is that seedling and adult plant responses are not completely correlated (Kema & van Silfhout, 1997). However, previous greenhouse studies using bulked isolates (Ahmed *et al.*, 1996) ranked seedlings of these cultivars the same as adult plants in the field.

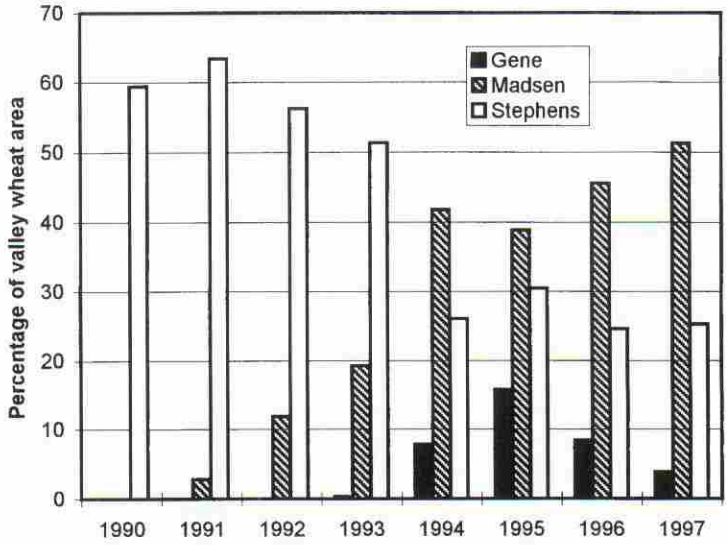

Figure 2 Percentage of soft white winter wheat area occupied by three cultivars in the Willamette Valley of Oregon during the period 1990–97. Data are compiled from successive editions of Oregon Agriculture & Fisheries Statistics, published by the US Department of Agriculture and the Oregon Department of Agriculture.

Kema & van Silfhout (1997) showed that adult plants were more susceptible than seedlings in a set of 22 cultivars each challenged with three individual *M. graminicola* isolates. While such developmental factors might explain the avirulence of Madsen and Stephens isolates on the Gene seedlings, they would not account for the consistent and specific virulence of Gene isolates on Gene that was observed.

It may be speculated why no isolates from Stephens and only one from Madsen possessed virulence to Gene. A possible explanation is that virulence to Gene entails a fitness cost under field competition that is not evident in single-isolate greenhouse experiments. Alternatively, isolates adapted to Gene may comprise a small proportion of the *M. graminicola* population, perhaps related to the relatively small area on which it was cultivated. In either case, isolates virulent to Gene would be detected more easily under the selective effect of that cultivar.

The data support the conclusion that specific cultivar–isolate interactions operate, although not exclusively, in the *M. graminicola/Triticum aestivum* pathosystem. The isolate–tester interaction was significant at $P = 0{\cdot}0979$ for the 1997 isolates. The intraclass correlations estimated for the 1997 replicates were consistently about one-third from the top of the range, and their 95% confidence intervals overlapped substantially. This implies low to moderate specificity in our total set of isolates and testers, which is consistent with the finding of a lack of specificity to Madsen and Stephens, and specificity to Gene.

The specificity detected in an agricultural host–pathogen system will depend greatly on the nature of resistance that is commercially deployed. Gene was the first wheat cultivar with high resistance to septoria tritici leaf blotch to be grown commercially in the Willamette Valley of Oregon, and it was possible to identify cultivar-specific isolates within 5 years of its release.

Detection of clear specificity only after use of highly resistant cultivars has occurred previously in other host–pathogen systems. For example, specificity in the rice (*Oryzae sativa*)/*Xanthomonas oryzae* pv. *oryzae* system was debated into the 1970s. However, the deployment and subsequent breakdown of resistant rice cultivars in Japan and the Philippines in the 1970s resulted in the clear identification of physiological races (Mew, 1987). Subsequently, bacterial blight of rice has become one of the best molecularly characterized gene-for-gene systems (Hopkins *et al.*, 1992; Song *et al.*, 1995).

In agreement with Kema & van Silfhout (1997), it is suggested that the slow evolution of virulence in *M. graminicola* that has often been observed results, in part, from the incomplete nature of most resistances that have been used. However, it is anticipated that qualitative resistance to *M. graminicola* will be no more durable than in any other host–pathogen system, as illustrated by the performance of Gene in the Willamette Valley of Oregon.

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